

ORIGINAL ARTICLE

# Induction of purple sulfur bacterial growth in dairy wastewater lagoons by circulation

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## Keywords

carotenoid pigments, circulation, dairy manure, purple sulfur bacteria, wastewater.

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## Abstract

**Aims:** To determine whether circulation of dairy wastewater induces the growth of phototrophic purple sulfur bacteria (PSB).

**Methods and Results:** Two dairy wastewater lagoons that were similar in size, geographic location, number and type of cattle loading the lagoons were chosen. The only obvious visual difference between them was that one was stagnant and the water was brown in colour (Farm 1), and the other was circulated and the water was red in colour because of the presence of PSB that contained carotenoid pigments (Farm 2). Both wastewaters were sampled monthly for 3 months and assayed for PSB and extractable carotenoid pigments (ECP). After this point, circulators were placed in the wastewater lagoon on Farm 1, and samples were taken monthly for 9 months and assayed for PSB and ECP. Before the installation of circulators, no PSB-like 16S rRNA sequences or ECP were observed in the wastewater from Farm 1; however, both were observed in the wastewater from Farm 2. After the installation of circulators, statistically greater levels of PSB and extractable carotenoid pigments were observed in the wastewater from Farm 1.

**Conclusions:** Circulation enhances the growth of PSB in dairy wastewater.

**Significance and Impact of this Study:** Because PSB utilize H<sub>2</sub>S and volatile organic acids (VOA) as an electron source for photosynthesis, and VOA and alcohols as a carbon source for growth, the increase in these bacteria should reduce H<sub>2</sub>S, volatile organic compounds and alcohol emissions from the lagoons, enhancing the air quality in dairy farming areas.

## Introduction

California is the largest dairy producing state in the United States with >1.8 million lactating cows housed on c. 2000 dairies, most of which are located in the San Joaquin Valley (Agricultural Statistics Board 2007). The average dairy in California houses c. 900 cows, which reside in free-stall barns where they urinate and defecate on cement floors. A typical 450-kg dairy cow produces c. 40 kg of waste a day (Miner *et al.* 2000); thus, the average California dairy produces 36 thousand kg of waste a day or 13 million kg of waste a year. To efficiently manage these large amounts of waste, dairies commonly employ a

hydraulic waste removal system that flushes water across the floors to remove the waste. The flush water and waste flow into a solid/liquid separator that removes the large particulate matter from the waste stream, while the liquid portion of the waste is pumped into large holding lagoons and is reused as the flush water to remove waste from the free-stall barn floors.

Three or four times a year, wastewater from the lagoons is mixed with fresh irrigation water and applied to croplands surrounding the dairy as a fertiliser for crops destined for both human and animal consumption. This practice is of concern because pathogenic bacteria, including *Escherichia coli* O157:H7 (Hancock *et al.* 1998),

*Salmonella* sp. (Warnick *et al.* 2001) and *Campylobacter* sp. (Wesley *et al.* 2000), have been associated with dairy waste and may contaminate the crops (Cieslak *et al.* 1993; Pell 1997; Natvig *et al.* 2002). In addition to pathogenic bacteria, undesirable chemicals such as sodium chloride, phosphate and nitrate can build up in the soils and leach into surface and ground waters rendering them unsuitable for human or animal consumption (Krapac *et al.* 1998; Nunez-Delgado *et al.* 2002; Shomar *et al.* 2008).

Another problem caused by the vast amounts of waste and wastewater is the volatilization of odorous and harmful chemicals such as hydrogen sulfide and volatile organic compounds (mostly alcohols and volatile organic acids) (Warnick *et al.* 2001; Shaw *et al.* 2007; Son *et al.* 2008). The greatest concentration of dairy cattle in California resides within the San Joaquin Valley, which is surrounded by mountain ranges on three sides. This unique 'bowl-like' geography restricts air movement and has caused the area to have some of the worst air quality in the United States (Schwehr 2004). One possible way to reduce the volatilization of hydrogen sulfide and volatile organic compounds is to induce the growth of photosynthetic purple sulfur bacteria (PSB) (family *Chromatiaceae*) within the wastewater lagoons. Several studies have shown that PSB can grow photoheterotrophically utilizing hydrogen sulfide and various volatile organic acids (VOA) as electron donors for photosynthesis as well as VOA and alcohols as a carbon source for growth (Dilling *et al.* 1995; Guyoneaud *et al.* 1998; Imhoff *et al.* 1998; Zarr *et al.* 2003).

Previously, we compared the bacterial population structure of circulated and stagnant wastewater lagoons and noted that the PSB were the predominant phototrophic bacteria within the lagoons. We also observed that the circulated lagoons contained significantly greater percentages of PSB than the stagnant lagoons (McGarvey *et al.* 2005). In the present study, we determined whether we could induce PSB blooms by installing circulators in a previously stagnant wastewater lagoon that contained undetectable levels of PSB by 16S rRNA gene sequence analysis. To accomplish this, we examined the percentages of PSB-like 16S rRNA gene sequences in libraries derived from a stagnant lagoon once a month for 3 months. We then installed circulators into the stagnant lagoon and examined the percentage of PSB-like 16S rRNA gene sequences derived from this lagoon for an additional 9 months. As a positive control, we also sampled from a circulated wastewater lagoon that had been known to have a consistent PSB bloom for many years. In addition to 16S rRNA sequence analysis, we extracted carotenoid pigments from both wastewaters to determine whether carotenoid pigment extraction could be used as a simple, low cost method to determine PSB's presence.

## Materials and methods

### Wastewater lagoons

A stagnant dairy wastewater lagoon (Farm 1) that had never been known to have a purple sulfur bacterial bloom by 16S rRNA gene sequence analysis and a circulated dairy wastewater lagoon (Farm 2) that had previously been identified as having a persistent PSB bloom (McGarvey, unpublished data) were selected for this study. Both wastewater lagoons were located within the greater Modesto, CA area (30 km radius of Modesto, CA, USA), ensuring that both lagoons received similar environmental conditions (i.e. fluctuations in temperature, ambient light, rain fall, etc.). Both lagoons were rectangular in shape, c. 4.5 m in depth, and contained c. 16 million litre of wastewater. In addition, both lagoons received waste from c. the same number of Holstein milking cows (c. 900) fed similar diets formulated by an animal nutritionist, ensuring similar loading rates. The only obvious difference between the two wastewater lagoons was that one used circulators and the other did not. After 3 months of sampling (January 2007), circulators (one floating, 3 hp, upflow circulator per 4 million litre) were installed in the wastewater lagoon on Farm 1 to achieve partial mixing of the wastewater.

### Sample collection and preparation

Wastewater samples were taken from the wastewater lagoons on Farm 1 (stagnant) and Farm 2 (circulated) monthly from 11 January 2006 until 10 January 2007. After the third sampling period (1 January 2007), four circulators (Absolute Aeration, Lexington, NE, USA) were installed in the wastewater lagoon on Farm 1, and samples were taken monthly for the next 9 months. One litre wastewater samples were taken from the hydraulic flush system that pumps wastewater from the bottom 25% of the lagoon (c. 3 m from the top) by starting the flush cycle and allowing the water to run for 2 min to clear the line. A sterile 2-l container was placed in the path of the flush and filled half full. Samples were transported to the Western Regional Research Center in Albany, CA, stored overnight at 4°C and processed the next day.

### Viable counts of bacteria and chemical analysis of wastewater

Samples were quantified for viable bacteria by performing serial dilutions in phosphate-buffered saline that was vortex-agitated for 2 min, plated onto nutrient agar plates and incubated at 25°C for 3 days under normal atmospheric conditions or in an anaerobic chamber. To

quantify the number of coliform bacteria, samples were diluted as described earlier, plated onto MacConkey agar plates and incubated at 37°C for 18 h. All media were purchased from Difco (Detroit, MI, USA) as dehydrated powders. Chemical analysis was performed using protocols from the Standard Methods of the American Public Health Association (1995).

#### DNA extraction from wastewater samples

From each wastewater sample, two 5 ml subsamples were taken for DNA extraction. DNA was extracted from the subsamples using a modification of the MoBio UltraClean Fecal DNA isolation kit (MoBio, Solano Beach, CA, USA) as described previously (McGarvey *et al.* 2007). Each subsample was used to generate 96 16S rRNA sequence clones for 192 clones per wastewater sample.

#### PCR amplification of 16S rRNA genes and library construction

PCR amplification 16S rRNA genes was carried out as described previously (McGarvey *et al.* 2007) using the bacterial 16S rRNA gene-specific primers 27f (5'-AGA-GTTTGATCCTGGCTCAG-3') and 1392r (5'-GAC-GGGCGGTGTGTAC-3') (Lane 1991). PCRs were performed as recommended by Polz and Cavanaugh (1998) to reduce bias in amplification. A PCR and cloning experiment were performed for each subsample DNA, and 96 clones were picked from each PCR (192 total for each sample) to minimize potential PCR bias.

#### DNA template preparation and sequencing

DNA templates were prepared using the TempliPhi HT amplification kit (GE Healthcare, Piscataway, NJ, USA) as per the manufacturer's instructions. Sequencing reactions were performed using the primer 1392r and the BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were purified using the BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA); electrophoresis and readout were performed using an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Two 96-well plates of 16S rDNA sequences were analysed for each sample; in total, 2304 sequences were analysed from each lagoon over the 1-year period.

#### DNA sequence analysis

DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5' and the 3' ends

using the SEQMAN software (ver. 7.2; DNASTAR Inc. Madison, WI, USA). Only sequences with unambiguous reads >500 bp were used; each read c. 600 bp. The predicted 16S rDNA sequences from were compared to 16S rDNA sequences in a BLASTable database as described previously (McGarvey *et al.* 2007). Operational taxonomic units (OTUs) were defined as clones with >97% sequence identity. OTUs belonging to the family *Chromatiaceae* were identified using the CLASSIFIER software available at <http://rdp.cme.msu.edu/classifier/classifier.jsp>. Complete 16S rRNA libraries were compared to each other using the LIBRARY COMPARE software available at <http://rdp.cme.msu.edu/comparison/comp.jsp>. Sequences of the two most commonly isolated PSB-like 16S rRNA genes were submitted to GenBank under the BankIt numbers 1138951 and 1138953.

#### Statistical methods

The percent coverage of the total OTUs identified in each sample was calculated using the equation  $C = 1 - (n/N) \times 100$  where  $C$  is the percentage coverage,  $n$  is the number of singleton sequences, and  $N$  is the number of clones examined (Good 1953). The members of the family *Chromatiaceae* were identified using the CLASSIFIER software (Cole *et al.* 2003). The percentage of family *Chromatiaceae* in one library was considered significantly different from another library if both statistical methods (Student's  $t$ -test and LIBRARY COMPARE) were in agreement. All 16S rRNA sequences were subjected to Chimaera Check available on the Ribosomal Database Project website.

#### Extraction and quantification of carotenoid pigments from wastewater

The method for carotenoid pigment extraction and quantification used was described previously (McGarvey *et al.* 2005). Briefly, 1 ml of wastewater was centrifuged at 14 000  $g$  for 10 min to pellet the bacteria in the wastewater. The supernatant was removed, and the pellet was suspended in 1 ml of methanol and extracted overnight at 4°C. The methanol extract was centrifuged at 14 000  $g$  for 10 min, the supernatant discarded, and the pellet suspended in 100  $\mu$ l of methanol and centrifuged at 14 000  $g$  for 10 min. The methanol supernatant was discarded, the pellet was suspended in 1 ml of ethyl acetate (EtOAc) and allowed to extract overnight at 4°C, centrifuged at 14 000  $g$  for 10 min, and the supernatant was removed, saved and analysed using UV/Vis spectrometry. The concentration of carotenoid pigments within the extracts was approximated to mg spirilloxanthin  $l^{-1}$  by dividing the observed absorbance maximum (OD492 nm) of the

**Table 1** Aerobic, anaerobic and coliform plate counts of wastewaters

	Farm 1 (SD)	Farm 2 (SD)
Aerobic plate counts (CFU ml <sup>-1</sup> )		
Before*	<b>1.6 × 10<sup>6</sup></b> (2.3 × 10 <sup>6</sup> )	1.2 × 10 <sup>6</sup> (1.9 × 10 <sup>6</sup> )
After†	<b>6.0 × 10<sup>6</sup></b> (2.3 × 10 <sup>6</sup> )	1.9 × 10 <sup>6</sup> (1.3 × 10 <sup>6</sup> )
Anaerobic plate counts (CFU ml <sup>-1</sup> )		
Before	1.3 × 10 <sup>5</sup> (1.7 × 10 <sup>5</sup> )	7.9 × 10 <sup>4</sup> (1.1 × 10 <sup>5</sup> )
After	1.0 × 10 <sup>6</sup> (1.6 × 10 <sup>6</sup> )	1.8 × 10 <sup>5</sup> (1.4 × 10 <sup>5</sup> )
Coliform plate counts (CFU ml <sup>-1</sup> )		
Before	2.2 × 10 <sup>3</sup> (2.7 × 10 <sup>3</sup> )	9.9 × 10 <sup>2</sup> (1.2 × 10 <sup>3</sup> )
After	2.7 × 10 <sup>3</sup> (1.7 × 10 <sup>3</sup> )	2.6 × 10 <sup>3</sup> (2.6 × 10 <sup>3</sup> )

Significant differences ( $P < 0.05$ ) are in bold.

\*Before the installation of circulators on Farm 1.

†After the installation of circulators on Farm 1.

EtOAc extract by the molar extinction coefficient of spirilloxanthin at 492 nm (97 mmol l<sup>-1</sup> cm<sup>-1</sup>). The limit of detection of spirilloxanthin in wastewater was 0.01 mg l<sup>-1</sup>.

## Results

### Cultural, chemical and physical analyses of dairy wastewater lagoons

Wastewater samples taken monthly for a 1-year period revealed fluctuations in the numbers of aerobic, anaerobic and coliform plate counts from month to month (Table 1). Significantly, greater numbers of aerobic bacteria were observed in the wastewater from Farm 1 after the installation of circulators ( $P < 0.05$ ). No significant change in the number of aerobic bacteria was observed in the wastewater from Farm 2 between these time points. No significant differences were observed in the anaerobic or coliform plate counts after the installation of circulators on Farm 1 or on Farm 2 between these time points. Of the chemical and physical parameters measured, the wastewaters from Farm 1 had a significantly greater

concentration of NO<sub>3</sub> and significantly lower concentration of P after the installation of circulators (Table 2). No significant differences in any of the parameters tested were observed in the wastewater from Farm 2 between these time points. No significant difference in wastewater temperatures were observed and ranged from 12 to 21°C.

### Analysis of 16S rRNA sequence libraries

The level of operational taxonomic unit (OTU) coverage in the wastewaters was estimated to be 88.2% for Farm 1 and 80.0% for Farm 2 (data not shown). Analysis of the percentage of 16S rRNA sequences assigned to the PSB before and after the installation of circulators on Farm 1 revealed a significant increase in these sequences (undetectable *vs* 0.9%), while the percentage of these sequences in the wastewater from Farm 2 remained statistically unchanged (1.1 *vs* 1.5%) (Table 3).

### Concentration of carotenoid pigments in wastewaters

The concentration of extractable carotenoid pigments in the wastewaters was measured monthly (Table 4). Before the installation of circulators on Farm 1, no extractable carotenoid pigments were observed in the wastewater; however, after the installation of circulators, a significant increase in extractable pigments was observed (undetectable *vs* 0.11 mg spirilloxanthin l<sup>-1</sup>). Between these same periods, the wastewater from Farm 2 had a significant decrease in the level of extractable carotenoid pigments (1.70 *vs* 0.82 mg spirilloxanthin l<sup>-1</sup>).

## Discussion

In a previous study, circulation was shown to have little effect on the gross biological, chemical and physical parameters of dairy wastewater lagoons (McGarvey *et al.* 2005). This is likely because of the high level of loading that a typical California dairy wastewater lagoon receives

**Table 2** Chemical and physical analysis of wastewaters

	TKN(SD)	NH <sub>4</sub> (SD)	NO <sub>3</sub> (SD)	SO <sub>4</sub> (SD)	P (SD)	K (SD)	Na (SD)	TS (SD)	COD (SD)
Farm 1									
Before*	409 (33.4)	249 (7.8)	<b>0.2</b> (0.4)	34.3 (2.1)	<b>96.8</b> (17.6)	653 (108)	145 (21.0)	3467 (603)	3613 (1297)
After	339 (82.6)	231 (51.7)	<b>1.1</b> (0.5)	54.4 (22.3)	<b>61.1</b> (59.8)	502 (122)	111 (37.0)	2633 (856)	2329 (1490)
Farm 2									
Before	431 (48.0)	283 (21.4)	1.7 (0.9)	90.7 (14.4)	53.2 (2.2)	654 (83.3)	185 (24.0)	4033 (551)	3800 (854)
After	381 (88.8)	250 (55.8)	1.2 (1.0)	83.4 (16.9)	61.2 (9.4)	618 (152)	169 (45.6)	3238 (1283)	2992 (1240)

All measurements are in mg l<sup>-1</sup> (ppm).

Significant differences ( $P < 0.05$ ) in bold.

TKN, total Kjeldahl nitrogen; TS, total solids; COD, chemical oxygen demand.

\*Before the installation of circulators on Farm 1.

**Table 3** Percentage of 16S rRNA gene sequences assigned to the family *Chromatiaceae*

Month	Farm 1	Farm 2
November	0.0	0.7
December	0.0	1.4
January	0.0	1.3
Avg. (SD)	<b>0.0 (0.0)</b>	1.1 (0.4)
Installation of circulators on Farm 1		
February	0.0	1.3
March	0.0	1.9
April	0.6	1.5
May	0.5	1.1
June	0.6	1.3
July	1.7	1.8
August	1.6	ND*
September	1.7	1.5
October	1.6	1.4
Avg. (SD)	<b>0.9 (0.7)</b>	1.5 (0.3)

Level of detection = 0.17%.

Significant differences ( $P < 0.05$ ) in bold.

ND, not determined for that month.

**Table 4** Concentration of carotenoid pigments extracted from wastewaters

Date	Farm 1	Farm 2
November 2006	0.0	1.73
December 2006	0.0	1.63
January 2007	0.0	1.75
Avg. (SD)	<b>0.0 (0.0)</b>	<b>1.70 (0.06)</b>
Installation of circulators on Farm 1		
February 2007	0.0	1.27
March 2007	0.0	1.00
April 2007	0.11	0.50
May 2007	0.13	1.03
June 2007	0.15	1.08
July 2007	0.37	0.27
August 2007	0.20	ND
September 2007	0.21	0.71
October 2007	0.21	0.69
Avg. (SD)	<b>0.11 (0.13)</b>	<b>0.82 (0.33)</b>

Data reported as mg spirilloxanthin  $l^{-1}$  wastewater.

Limit of detection = 0.01 mg spirilloxanthin  $l^{-1}$  wastewater.

Significant differences ( $P < 0.05$ ) in bold.

ND, not determined for that month.

[c. 18 000 kg waste  $day^{-1}$ , assuming 50% solid separation (Miner *et al.* 2000)]. The data from the present study is in agreement with these findings; however, we did observe a significant increase in the aerobic plate counts and the concentration of  $NO_3$  after the installation of circulators on Farm 1. These findings are consistent with the fact that circulation incorporates greater amounts of oxygen into the wastewater than stagnation; however, in previous

studies, we were only able to detect dissolved oxygen (DO) within a metre or so of the circulators. This is likely because of the rapid uptake of DO from the facultative organisms within the wastewater lagoons. We also observed a small but statistically significant decrease in the level of phosphorus after the installation of circulators on Farm 1, which brought the wastewater phosphorus concentration to a similar level as the circulated wastewater on Farm 2. The decrease in phosphorus may be caused by process termed 'anaerobic-anoxic enhanced biological phosphorus removal' ( $A_2$  EBPR) in which denitrifying organisms accumulate polyphosphate within the cell (Kuba *et al.* 1996), although it is also possible that mixing the wastewater allows the bacteria greater access to phosphorus and thus their ability to uptake this essential nutrient. In the previous study, circulating also had little effect on the most prevalent OTUs observed in dairy wastewater, which was likely because of the high level of bacteria in the waste the lagoons received. In the present study, we also observed this phenomenon with seven of ten of the most prevalent OTUs remaining as the most prevalent after the installation of circulators on Farm 1, and none of the OTUs disappearing completely after the installation of circulators (data not shown).

Both the prevalence of 16S rRNA sequences assigned to the family *Chromatiaceae* and the amount of extractable carotenoid pigments increased significantly after the installation of circulators in the wastewater lagoon on Farm 1. Before the installation of circulators, we were unable to identify any PSB-like 16S rRNA sequences in the wastewater from Farm 1; however, after the installation of circulators, these sequences increased to almost 2% of the total, a level similar to that observed in the circulated wastewater from Farm 2. Interestingly, no 16S rRNA gene sequences associated with other groups of photosynthetic bacteria, such as the purple non-sulfur bacteria were detected in significant amounts in either wastewater. Concurrent with the increase in PSB-like 16S rRNA sequences was a significant increase in the level of extractable carotenoid pigments (ECP) in the wastewater lagoon on Farm 1. Although the average levels were lower than those observed on Farm 2 (0.16 mg  $l^{-1}$  vs 0.82 mg  $l^{-1}$ ), most of this difference can be explained by the fact that for the first few months after the installation of circulators no or very low levels of ECP were observed in the wastewater from Farm 1. However, after this period, ECP were consistently isolated (Table 3). We also observed a small but significant decrease in the levels of ECP from the wastewaters from Farm 2 during the spring and summer months of 2007. It is possible that environmental conditions during the spring and summer resulted in lower levels of pigment production; however, we have no data to confirm this hypothesis.

From these data we conclude that circulation enhances the growth of phototrophic PSB (family *Chromatiaceae*) within dairy wastewater lagoons. It is not clear how circulation enhances the growth of these bacteria, but several possibilities exist. First, circulation could expose these phototrophic bacteria to greater amounts of light resulting from the constant movement of the water. Secondly, circulation should destroy any stratification because of chemical and thermal gradients in the wastewater lagoons, exposing the bacteria to greater levels of nutrients, which might otherwise be sequestered below the phototrophic zone. Lastly, circulation of dairy wastewater tends to remove any floating debris that can form a cap on top of the lagoon that can block sunlight from entering the wastewater. Regardless of the method, circulation was correlated with the induction of PSB growth. Because PSB utilize both H<sub>2</sub>S and volatile fatty acids as a source of electrons for photosynthesis and volatile fatty acids as a carbon source for growth (Holm and Vennes 1970; Do et al. 2003; van der Steen et al. 2003), the presence of these bacteria within wastewater lagoons has been shown to reduce the rates of volatile organic compounds emissions (Dilling et al. 1995) and thus should be a benefit to both the dairy industry and the residents of dairy farming areas. Although we did not perform quantitative measurements of odour release from the wastewaters, we have consistently noted that circulated wastewater lagoons are less malodorous than stagnant lagoons, which is also a benefit to the residents of agricultural areas.

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